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Award Number: W81XWH-10-1-0214

TITLE: The Biological and Clinical Significance of Androgen
Receptor Variants

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REPORT DATE: April 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE April 2014		2. REPORT TYPE Final		3. DATES COVERED 1 APR 2010 - 31 MAR 2014	
4. TITLE AND SUBTITLE The Biological and Clinical Significance of Androgen Receptor Variants				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0214	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Donna M. Peehl email: dpeehl@stanford.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Leland Stanford Junior University Stanford, CA 94305				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We achieved all of aims proposed for this project. Aim 1 was to evaluate AR-V7 as a prognostic marker in high-grade prostate cancer (PCa). We found that AR-V7 levels did not predict biochemical recurrence (BCR). Aim 2 was to determine whether AR-V7 levels in low-grade PCa were prognostic, or predicted concurrent presence of high-grade PCa. We found that neither was the case. However, we did find that the ratio of AR-V1:AR-V7 was higher in low- grade PCa without concurrent high-grade PCa. Since AR-V1 reportedly blocks constitutive androgen signaling of AR-V7, this suggests that activity of AR-V7 in low-grade PCa might be associated with development of high-grade Pea. Aim 3 was to evaluate the role of AR-V7 in mediating primary castration-resistant PCa (CRPC) in a tumorgraft model. We successfully established tumorgrafts from primary PCa and observed the development of CRPC and androgen- independent gene expression; however, we were unable to correlate AR-V7 levels with development of CRPC.					
15. SUBJECT TERMS- prostate cancer, androgen receptor, prognostic biomarkers					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 28	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Of the >20 androgen receptor (AR) splicing variants currently identified, AR-V7 is the best-characterized variant in clinical samples as well as in experimental models. Strong evidence has emerged that AR-V7 plays an important role in supporting the castration-resistant prostate cancer (CRPC) phenotype, and therefore may serve as a prognostic marker and therapeutic target. Specifically, quantitative real-time polymerase chain reaction (qRT-PCR) showed an average 20-fold higher AR-V7 transcript level in CRPC vs. hormone naïve PCa.¹ Immunohistochemistry using AR-V7-specific antibody also demonstrated elevated protein expression of AR-V7 in CRPC vs. hormone naïve prostate cancer (PCa).² In addition, AR-V7 mRNA expression in bone metastases of men with CRPC was associated with disturbed cell cycle regulation and short survival.³ Functional studies in laboratories confirmed the ability of AR-V7 to support androgen-independent growth of LNCaP cells in vitro and as xenografts in vivo.¹ Moreover, Hu et al. demonstrated that suppression of ligand-mediated full-length AR signaling leads to increased AR variant expression including AR-V7 in two cell line models of CRPC.⁴ This increased expression of AR variants activated a distinct expression signature enriched for cell cycle genes, suggesting an important mechanism of drug resistance to androgen ablation therapy. These results suggest that androgen ablation may upregulate AR-V7 expression in men receiving hormonal therapy, enabling the bypass of androgen dependence in these patients. However, the mechanism of AR-V7 function is not clear, although recent studies have implicated other AR splice variants and full-length AR in the process.⁵ In particular, AR-V1 has been shown to interfere with AR-V7 activity when co-expressed in DU 145 cells.⁵

The first objective of our proposed research was to investigate the biological and clinical roles of AR-V7 in hormone-naïve primary adenocarcinomas of the prostate by evaluating the relationship of AR-V7 expression and Gleason grade, and determining whether AR-V7, AR-V1, or their ratio is a prognostic marker in patients with indeterminant risk of progression, i.e., men with 30-70% grade 4/5 having a risk of progression of ~50%. Our second objective was to determine whether AR-V7 expression is upregulated upon androgen depletion, enabling androgen-independent expression of AR target genes in human PCa using a new “tumorgraft” in vivo model of primary adenocarcinomas. The aims of our project were to (1) evaluate AR-V7, AR-V1, or their ratio as a prognostic marker in cancers of Gleason grades 4 and 5, (2) evaluate AR-V7, AR-V1, or their ratio in grade 3 cancer as a prognostic marker or surrogate marker for concurrent presence of grade 4/5 cancer, and (3) evaluate the ability of AR-V7 to mediate androgen-independent gene expression in vivo.

BODY

Our first designated task was to evaluate AR-V7 or AR-V1 or their ratio as a prognostic marker in cancers with both high-grade (grade 4/5) and low-grade (grade 3) components and of indeterminant risk of progression (months 1-18). Our specific goals were to (a) select and microdissect grade 4/5 and grade 3 cancers from formalin-fixed, paraffin-embedded (FFPE) tissue blocks of radical prostatectomy cases, (b) measure AR-V7 and AR-V1 mRNA levels in grade 4/5 and grade 3 cancers separately using branched DNA (bDNA) assay, and (c) correlate

AR-V7, AR-V1, and their ratio with histomorphologic and clinical variables [% grade 4/5, tumor volume, age, and preoperative prostate-specific antigen (PSA)]. We accomplished all of the specific goals of this aim. We demonstrated that in this mixed-grade cohort: (1) Expression of AR-V7 and AR-V1 was associated within the same Gleason grades across cases but not across cancer grades within the same prostate (see Fig. 1 in Zhao et al., J. Urol. 188:2158-2164, 2012); (2) AR-V7 expression in both grade 3 and grade 4/5 cancers in this cohort inversely correlated with pre-operative serum PSA (see Table 2 in Zhao et al., J. Urol. 188:2158-2164, 2012); and (3) Neither AR-V7, AR-V1 nor AR-V7/AR-V1 levels predicted risk of recurrence (see Fig. 3 in Zhao et al., J. Urol. 188:2158-2164, 2012).

Our second aim was to evaluate AR-V7 or AR-V1 or their ratio in cancers with 100% grade 3 and compare to those in grade 3 cancers with concurrent grade 4/5 cancers (months 18 – 24). Our specific goals were to (a) select and microdissect cancers from cases with 100% grade 3 cancer, (b) analyze expression levels of AR-V7 and AR-V1 in grade 3 cancers, and (c) correlate AR-V7, AR-V1, or their ratio with histomorphologic and clinical variables and compare them with those in grade 3 cancer with concurrent grade 4/5 cancer analyzed in Aim 1. We accomplished all of the specific goals of this aim. We demonstrated that: (1) AR-V1 expression in the grade 3-only cohort was significantly higher than that in grade 3 or grade 4/5 cancers in the mixed-grade cohort and the AR-V7/AR-V1 ratio in the grade 3-only cohort was lower than that in grade 3 cancer with associated grade 4/5 cancer, suggesting that grade 4/5 cancer and grade 3 cancer with associated grade 4/5 cancer have higher levels of AR-V7 function than grade 3-only cancer, since AR-V1 supposedly interferes with AR-V7 function (see Fig. 2 in Zhao et al., J. Urol. 188:2158-2164, 2012); (2) AR-V7 expression in grade 3 cancer showed a significant positive association with index (largest) cancer volume (see Table 2 in Zhao et al., J. Urol. 188:2158-2164, 2012); (3) AR-V7 and AR-V1 expression inversely correlated with pre-operative serum PSA (see Table 2 in Zhao et al., J. Urol. 188:2158-2164, 2012).

Our third aim was to evaluate of the ability of AR-V7 to mediate androgen-independent gene expression in vivo using a tissue slice tumorgraft (TST) model (months 15-36). The specific goals of this aim were: (a) establishment of AR-V7-high and -low TSTs and (b) determination of response of cancers in TSTs with AR-V7 to androgen-deprivation. We accomplished these goals. Specifically, for (a), we established TSTs from 6 high-risk primary PCa by implanting precision-cut slices of tumor tissues under the renal capsule of γ c-/RAG2-mice that were implanted with a testosterone pellet to raise murine levels of testosterone to those typical of human males (see Tables 1 and 3 and Figs. 1, 2 and 3 in Zhao et al., J. Transl. Med. 11:199, 2013). We analyzed AR-V7 and AR-V1 expression in 4 of the 6 primary PCa specimens using bDNA technology and observed differential expression of AR-V7 in those cases. For (b), we first established TSTs in intact mice. One month after implantation, the experimental mice were castrated and the testosterone pellets were removed. The control mice were left intact with the pellet in place. One month later, the mice were sacrificed and the kidneys carrying the TSTs were retrieved and fixed in formalin. For all six cases, 3-5 TSTs in castrated and control groups were harvested and fixed in formalin for further analysis. TSTs from 2 of the cases showed complete pathologic regression in response to castration. For the other cases, cancer remained

after castration and these were considered to be CRPC (see **Figs. 3-7** in Zhao et al., J. Transl. Med. 11:199, 2013). Whether the TSTs were hormone-sensitive or castrate-resistant did not correlate with levels of AR-V7 in the original tissues; however, the number of cases was small and not sufficiently powered for statistical analysis.

We would have liked to determine whether AR-V7 expression increased in TSTs after castration, but the amount of tissue was too small to microdissect cancer and carry out molecular analysis. Instead, we attempted to evaluate AR-V7 levels by immunohistochemistry. We tested two antibodies purportedly specific for AR-V7: 1) a mouse monoclonal antibody available from A&G Precision Antibody™ (Columbia, MD);¹ and 2) a mouse monoclonal antibody developed using peptide sequences specific to AR-V7 (CKHLKMTRP3) by the Luo lab at John Hopkins University.⁴ The first antibody against AR-V7 did not appear to yield specific or appropriate staining. We then obtained the second antibody as a gift from Dr. Luo and tested on AR-V7-high and -low cases from the mixed-grade cohort in our lab. We used a monoclonal antibody against AR as a positive control and no primary antibody as a negative control. As for the first antibody, there were inappropriate staining patterns and there was no correlation between signal intensity of this antibody to RNA levels determined by bDNA assay. Considering these diverse (and inappropriate) staining patterns, we suspect non-specific antibody binding. We also attempted a sensitive fluorescence-based in situ hybridization (ISH) method recently developed by Sepmre et al. to visualize short RNAs in FFPE tissue specimens.⁶ By using locked nucleic acid (LNA)-modified DNA probes, a class of bi-cyclical high-affinity RNA analogues, this method allows the detection of short RNAs typical in FFPE samples with high sensitivity. Moreover, this ISH method is compatible with IHC to enable the detection of RNA and protein markers in the same tissue section for colocalization and functional studies. Considering the low abundance of AR-V7, we incorporated tyramide signal amplification (TSA), an enzyme-mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target protein or nucleic acid sequence in situ. The signal amplification is conferred by the turnover of multiple tyramide substrates per peroxidase label, which increases detection sensitivity up to 100-fold, as compared with conventional avidin-biotinylated enzyme complex (ABC) procedures. We tested this protocol on four cases of the mixed-grade cohort. Despite extensive attempts at optimization, we could not eliminate nonspecific labeling.

KEY RESEARCH ACCOMPLISHMENTS

- Identified a sensitive method of measuring AR-V7 and AR-V1 mRNA expression in FFPE samples using branched DNA (bDNA)
- Determined AR-V7 and AR-V1 expression levels in grade 4/5 and grade 3 components of the same tumor separately in a cohort of 53 men (the mixed-grade cohort) using QuantiGene® FFPE bDNA assay
- Determined AR-V7 and AR-V1 expression levels in 100% grade 3 cancer in a cohort of 52 men (the grade 3-only cohort) using QuantiGene® FFPE bDNA assay
- Determined the association of AR-V7 and AR-V1 expression levels and their ratio with hisopathological and clinical parameters, and their value as a prognostic marker in both

cohorts

- Generated TSTs from 6 high-risk PCa specimens maintained either in control or castrated mice
- Established tumorgrafts from PCa with high vs low expression of AR-V7 and recovered the grafts from intact and castrated animals
- Determined that the expression level of AR-V7 in cancer prior to castration did not predict development of CRPC
- Tested antibodies against AR-V7 protein and concluded that specificity was not as desired
- Evaluated a sensitive method of measuring AR-V7 gene expression in FFPE samples using TSA-aided in situ hybridization but did not successfully optimize

REPORTABLE OUTCOMES

Zhao, H., Coram, M., Nolley, R., Reese, S.W., Young, S. and Peehl, D.M. Transcript levels of androgen receptor splice variants, AR-V1 or AR-V7, do not predict recurrence in prostate cancer patients with indeterminant risk of progression. *J. Urol.* 188:2158-2164, 2012 (PMID 23088973)

Zhao, H., Thong, A., Nolley, R., Reese, S.W., Santos, J., Ingels, A. and Peehl, D.M. Patient-derived tissue slice grafts accurately depict response of high-risk primary prostate cancer to androgen deprivation therapy. *J. Transl. Med.* 11:199, 2013 (PMID 23985008)

CONCLUSIONS

We achieved all of our aims. We conclude that AR-V7 expression, whether alone or as a ratio to AR-V1 expression, does not predict biochemical recurrence after radical prostatectomy, whether measured in the low-grade or high-grade components of each cancer. However, the ratio of AR-V1:AR-V7 expression is higher in cancers with 100% Gleason grade 3 in comparison to grade 3 cancers co-existing with high-grade (grade 4 and/or 5) cancers, suggesting that 100% grade 3 cancers have different biological properties. We demonstrated that tumorgrafts can be generated from primary prostate cancers and used to study the development of castration-resistant prostate cancer. Although we could not confirm a role for AR-V7 in the development of castration-resistance in this model, future analyses with more sensitive and specific methods of measurement of AR-V7 may be informative.

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APPENDICES

Zhao, H., Coram, M., Nolley, R., Reese, S.W., Young, S. and Peehl, D.M. Transcript levels of androgen receptor splice variants, AR-V1 or AR-V7, do not predict recurrence in prostate cancer patients with indeterminate risk of progression. *J. Urol.* 188:2158-2164, 2012 (PMID 23088973)

Zhao, H., Thong, A., Nolley, R., Reese, S.W., Santos, J., Ingels, A. and Peehl, D.M. Patient-derived tissue slice grafts accurately depict response of high-risk primary prostate cancer to androgen deprivation therapy. *J. Transl. Med.* 11:199, 2013 (PMID 23985008)

Transcript Levels of Androgen Receptor Variant AR-V1 or AR-V7 Do Not Predict Recurrence in Patients with Prostate Cancer at Indeterminate Risk for Progression

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Abbreviations and Acronyms

AR = androgen receptor
bDNA = branched chain DNA
CRPC = castration resistant prostate cancer
ERG = Ets related gene
FFPE = formalin fixed, paraffin embedded
PCa = prostate cancer
PSA = prostate specific antigen
TMPRSS2-ERG = type 2 transmembrane-bound serine protease-ERG

Submitted for publication March 27, 2012.

Study received institutional review board approval.

Supported by Department of Defense W81XWH-10-1-0214.

Supplementary material for this article can be obtained at <http://www.jurology.com>.

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For another article on a related topic see page 2410.

Purpose: AR-V7, a ligand independent splice variant of androgen receptor, may support the growth of castration resistant prostate cancer and have prognostic value. Another variant, AR-V1, interferes with AR-V7 activity. We investigated whether AR-V7 or V1 expression would predict biochemical recurrence in men at indeterminate (about 50%) risk for progression following radical prostatectomy.

Materials and Methods: AR-V7 and V1 transcripts in a mixed grade cohort of 53 men in whom cancer contained 30% to 70% Gleason grade 4/5 and in a grade 3 only cohort of 52 were measured using a branched chain DNA assay. Spearman rank correlations of the transcripts, and histomorphological and clinical variables were determined. AR-V7 and V1 levels were assessed as determinants of recurrence in the mixed grade cohort by logistic regression and survival analysis. The impact of TMPRSS2-ERG gene fusion on prognosis was also evaluated.

Results: Neither AR-V7 nor V1 levels in grade 3 or 4/5 cancer in the mixed grade cohort were associated with recurrence or time to recurrence. However, AR-V7 and V1 inversely correlated with serum prostate specific antigen and positively correlated with age. The AR-V1 level in grade 3 cancer in the grade 3 only cohort was higher than in grade 3 or grade 4/5 components of mixed grade cancer. TMPRSS2-ERG fusion was not associated with AR-V7, AR-V1 or recurrence but it was associated with the percent of grade 4/5 cancer.

Conclusions: The AR-V1 or V7 transcript level does not predict recurrence in patients with high grade prostate cancer at indeterminate risk for progression. Grade 3 cancer in mixed grade tumors may differ from 100% grade 3 cancer, at least in AR-V1 expression.

Key Words: prostate; prostatic neoplasms; receptors, androgen; neoplasm recurrence, local; prostate-specific antigen

LIGAND independent splice variants of AR were proposed to be partly responsible for CRPC growth because they are constitutively active regardless of the androgen level.¹ Of the more than 20 AR splice variants identified, the best characterized is AR-V7.² Higher transcript and protein AR-V7 levels were detected in CRPC vs hormone naïve PCa.^{2,3} AR-V7 expression pre-

dicted biochemical recurrence after prostatectomy in hormone naïve PCa^{2,3} and was associated with short survival in CRPC.⁴ These results distinguished AR-V7 as the only clinically validated, alternatively spliced AR mRNA that encodes functional protein rather than splicing intermediates or error products targeted for RNA decay. Another AR variant, AR-V1,

interferes with AR-V7 activity, possibly through retention of AR-V7 by AR-V1 in cytoplasm.⁵

We determined whether the expression of AR-V7 or V1, or their ratio in PCa could predict recurrence independent of current prognostic variables.^{6–8} We selected 53 men with 30% to 70% grade 4/5 cancer (the mixed grade cohort) from a unique cohort of men whose radical prostatectomy specimens had been subjected to precise quantitation of 8 morphological variables by 1 pathologist.⁹ Because these men are at indeterminate risk for progression (about 50%) and are similar to men diagnosed with Gleason score 7 in the general population, new prognostic markers are urgently needed for management. We compared AR-V7 and V1 expression in low grade (grade 3) and high grade (grades 4/5) components of each cancer in this cohort. This differs from most studies of the prognostic value of potential biomarkers, which do not distinguish low from high grade cancer. While it might be assumed that the activity of a biomarker in high grade cancer drives progression, to our knowledge this is unknown.

We also selected a cohort of 52 men who were matched to the mixed grade cohort and in whom cancer was 100% Gleason grade 3 (grade 3 only). AR-V7 and V1 expression in these tumors was compared to that in the grade 3 components of cancer with concurrent grade 4/5 in the mixed grade cohort. Finally, we determined whether the status of the TMPRSS2-ERG fusion gene, which is the most common genetic alteration in PCa,¹⁰ affected the prognostic value of AR-V7 and/or V1 in the mixed grade cohort since a previous study suggested that men in whom TMPRSS2-ERG is expressed have different prognostic factors from those without the fusion.¹¹

MATERIALS AND METHODS

Patient and Prostate Specimen Clinical and Histopathological Characteristics

Patient tissues were collected between 1993 and 2003 under an institutional review board approved protocol. Archival blocks containing grade 3, or grade 4 and/or 5 cancer were selected from each case and cut into 6, 10 μ m sections. Hematoxylin and eosin staining was done to identify cancer areas marked on serial unstained slides. Cancer tissue was scraped away from benign tissue for RNA quantitation.

Patient characteristics and histopathological/morphological variables were obtained from an existing database (supplementary tables 1 and 2, jurology.com). As previously defined,⁹ PSA (biochemical) failure was the outcome for logistic regression and survival analyses.¹²

bDNA Assay and Immunohistochemistry

bDNA assay was performed to determine AR-V7 and V1 transcript levels in FFPE tissues using the QuantiGene® Plex 2.0 platform.¹³ Several clinically proven diagnostic tests are based on bDNA technology.^{14–16} It is more reli-

able, reproducible and sensitive than quantitative real-time polymerase chain reaction for measuring gene expression in FFPE tissue.¹³

Briefly, tissue homogenates were prepared according to the QuantiGene Sample Processing Kit for FFPE Tissues (Affymetrix®). RNA quantitation using the QuantiGene 2.0 Reagent Systems (Affymetrix) was performed according to manufacturer recommendations. Gene expression was measured in 3 technical replicates of each biological sample. AR-V7 and V1 signals were normalized to the geometric mean of the 2 reference genes, splicing factor 3a and adenosine diphosphate-ribosylation factor-like 8B.¹⁰

A monoclonal antibody against ERG (Epitomics®) was used as previously described.¹⁷

Statistical Analysis

Statistical analysis was done using the R statistical computing environment (<http://www.R-project.org>).¹⁸ Cases with 100% grade 3 cancer were matched to the mixed grade cancer cohort using the fullmatch function from the optmatch library to minimize Euclidean distance on Z-transformed age, surgery date, PSA and PSA 10 ng/ml or greater indicators as matching variables. The Wilcoxon and t tests, survival analysis using Cox proportional hazards models and logistic regression were done in the usual ways. Correlations were calculated using Spearman rank correlation statistics.

RESULTS

AR-V7 and V1 Expression

In Gleason grade 3 and 4/5 components of each mixed grade cancer. Using the bDNA assay, we first determined AR-V7 and V1 transcript levels in the mixed grade cohort (supplementary tables 1 and 2, jurology.com). AR-V7 levels showed a wider range of variation among cancers of the same Gleason grade than AR-V1 (table 1 and fig. 1, A and B). There was no statistical difference between AR-V7 and V1 levels in grade 3 and 4/5 cancers ($p = 0.61$ and 0.18 , respectively). These results demonstrated that AR-V7 and V1 were present in grade 3 and 4/5

Table 1. AR-V7 and V1 expression in mixed grade cohort

	Highest	Lowest	Highest/Lowest	No. Under Detection*
AR-V7:				
Grade 3	0.2783	0.0009	309	5†
Grade 4/5	0.2011	0.0014	144	0
AR-V1:				
Grade 3	0.2631	0.0103	26	3†
Grade 4/5	0.3090	0.0334	9	0
AR-V7/V1:				
Grade 3	1.9727	0.1252	16	6‡
Grade 4/5	1.2706	0.0068	187	0

* Detection limit 200 or fewer transcripts.

† Assigned value close to lowest detectable values, ie 10^{-4} , for statistical analysis.

‡ Treated as missing and excluded from analysis.

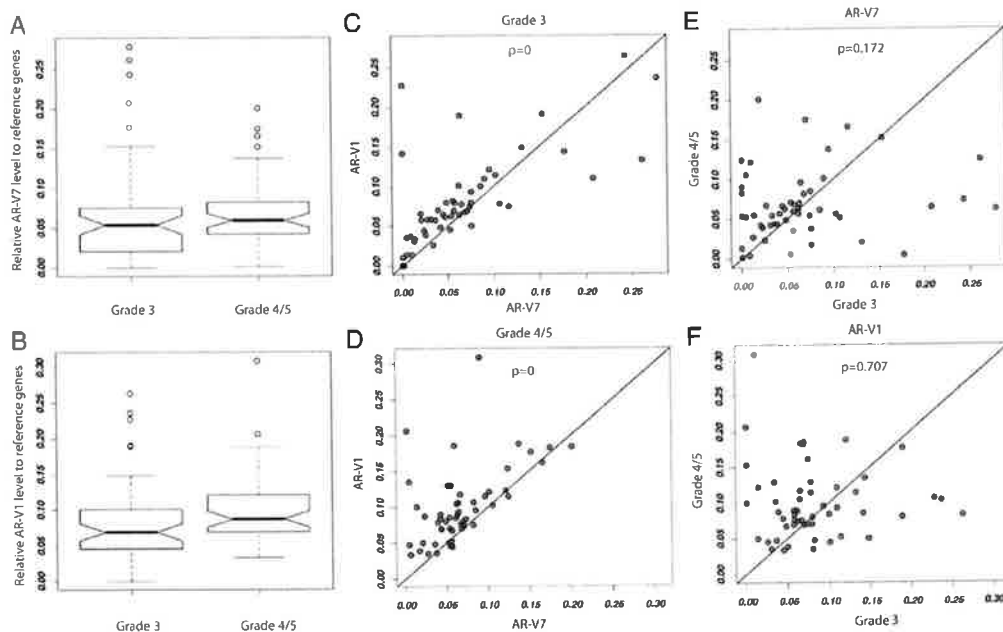


Figure 1. AR-V7 and V1 expression in grade 3 and 4/5 cancers in mixed grade cohort. Distribution of AR-V7 (A) and AR-V1 (B) expression. Expression of AR-V7 and V1 highly correlated in grade 3 (C) and 4/5 (D) cancers. AR-V7 (E) and AR-V1 (F) expression was not associated in grade 3 and 4/5 cancers.

components of the same cancer and levels did not significantly differ between the cancer grades.

The AR-V7/V1 ratio was much more variable in grade 4/5 than in grade 3 cancer components in this mixed grade cohort (table 1). Results suggest that the level of functional AR-V7, implied by the AR-V7/V1 ratio, was much more variable in high than in low grade cancer. However, the ratio did not statistically differ between grade 3 and grade 4/5 cancers ($p = 0.85$).

Association in same Gleason grades across tumors but not across grades in same tumor. In grade 3 and 4/5 cancers in the mixed grade cohort AR-V7 and V1 levels were significantly associated with each other (Spearman correlation coefficient 0.73 and 0.55, respectively, fig. 1, C and D). However, neither AR-V7 nor V1 expression in grade 3 cancer correlated with that in grade 4/5 cancer from the same case (fig. 1, E and F). In addition, the AR-V7/V1 ratio in grade 3 cancer did not correlate with that in grade 4/5 cancer from each case. Therefore, AR-V7 and V1 expression was associated in the same Gleason grades across cases but not across cancer grades in the same prostate.

AR-V1 Expression in Grade 3 Only Cohort Significantly Higher than in Grade 3 plus Associated Grade 4/5 Cancer

In the grade 3 only cohort AR-V7 expression was positively associated with AR-V1 expression ($p < 10^{-9}$,

supplementary table 1, jurology.com), as observed within grades in the mixed grade cohort (fig. 2, A). Interestingly, AR-V1 expression in the grade 3 only cohort was significantly higher than in grade 3 and 4/5 cancers in the mixed grade cohort ($p = 0.01$ and 0.02 , respectively, fig. 2, B). Moreover, the AR-V7/V1 ratio in the grade 3 only cohort was lower than in grade 3 cancer with associated grade 4/5 cancer and the difference was almost significant ($p = 0.07$). Results suggest that grade 4/5 and 3 cancers with associated grade 4/5 cancer have higher AR-V7 function than grade 3 only cancer since AR-V1 supposedly interferes with AR-V7 function.⁵

AR-V7 and V1 Expression

Inverse association with preoperative serum PSA in each cohort. On Spearman rank correlation analysis neither AR-V7 nor V1 correlated with prostate weight or the percent of grade 4/5 in the mixed grade or the grade 3 only cohort. However, AR-V7 expression in grade 3 cancer showed a significant positive association with the index (largest) cancer volume in each cohort (table 2). Moreover, AR-V7 expression in grade 3 cancer in each cohort and grade 4/5 cancer in the mixed grade cohort inversely correlated with preoperative serum PSA (table 2). In addition, in each cohort AR-V1 expression in grade 3 cancer inversely correlated with preoperative PSA (table 2). The AR-V7/V1 ratio in grade 4/5 cancer also inversely correlated with PSA. These findings

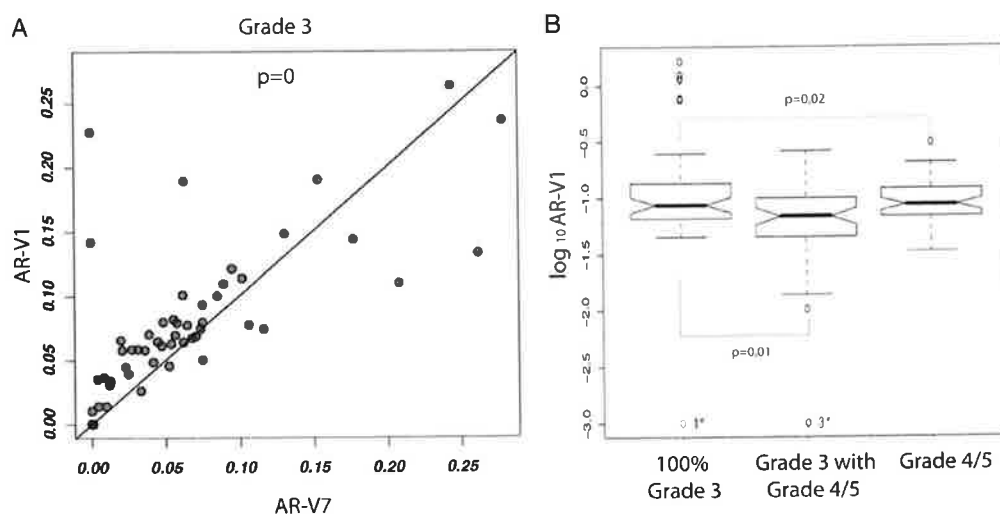


Figure 2. AR-V7 and V1 expression in grade 3 only cohort and comparison with mixed grade cohort. Expression of AR-V7 and V1 highly correlated in grade 3 only cancer (A). AR-V1 expression was significantly higher in grade 3 cancer in grade 3 only cohort compared to grade 3 and 4/5 cancers in mixed grade cohort (B).

demonstrated that AR-V7 and V1 expression in primary PCa was negatively associated with serum PSA.

Positive association with age in grade 4/5 cancer. AR-V7 and V1 expression in grade 4/5 but not grade 3 cancer in either cohort positively correlated with age (table 2). These findings suggest that with increased age AR-V7 and V1 increased in grade 4/5 but not grade 3 cancer regardless of whether there was associated grade 4/5 cancer.

Table 2. Significant association of AR-V7 and V1 with clinical and histopathological variables

	AR-V7 (grade)		AR-V1 (grade)	
	4	3	4	3
<i>Mixed grade cohort</i>				
PSA:*				
Rank correlation coefficient	-0.39	-0.31	—	-0.32
p Value	0.0048	0.023	—	0.019
Age:				
Rank correlation coefficient	0.34	—	0.40	—
p Value	0.012	—	0.0032	—
1st vol:				
Rank correlation coefficient	—	0.31	—	—
p Value	—	0.024	—	—
<i>Grade 3 only cohort</i>				
PSA:				
Rank correlation coefficient	—	-0.31	—	-0.32
p Value	—	0.022	—	0.021
1st Vol:				
Rank correlation coefficient	—	0.31	—	—
p Value	—	0.024	—	—

* For AR-V7/V1 grade 4 rank correlation coefficient -0.36 and $p = 0.0064$ with $p \leq 0.05$ for all values.

Not predictive of biochemical recurrence. No recurrence developed in the grade 3 only cohort. Of the 53 men in the mixed grade cohort 27 experienced recurrence at a median of 209 days during the median followup of 1,462 days. Of these cases 25 recurred within 2 years after prostatectomy. Logistic regression analysis revealed that neither AR-V7, AR-V1 nor AR-V7/V1 levels predicted the risk of recurrence (fig. 3). In addition, the splice variants did not predict time to recurrence on survival analysis using Cox proportional hazards models.

Impact of TMPRSS2-ERG Gene Fusion

TMPRSS2-ERG fusion gene status in mixed grade cancer was determined by immunohistochemistry against ERG (supplementary table 2, jurology.com).¹⁹ In 80% of cases ERG expression status was the same in grade 3 and 4/5 components of the same tumor, while in the other 20% status was different. This suggests that cells of tumors of different grades shared the same TMPRSS2-ERG gene fusion status in most cases.

Logistic regression analysis demonstrated that ERG expression was not associated with recurrence. In addition, AR-V7, AR-V1 and AR-V7/V1 were not prognostic regardless of fusion status. This suggests that TMPRSS2-ERG does not contribute to PCa prognosis in patients at indeterminate risk for recurrence alone or combined with AR-V7 and V1 expression. TMPRSS2-ERG status did not correlate with AR-V7 or V1 expression in grade 3 or 4/5 cancer according to the Spearman rank correlation. However, TMPRSS2-ERG expression positively correlated with the percent of grade 4/5.

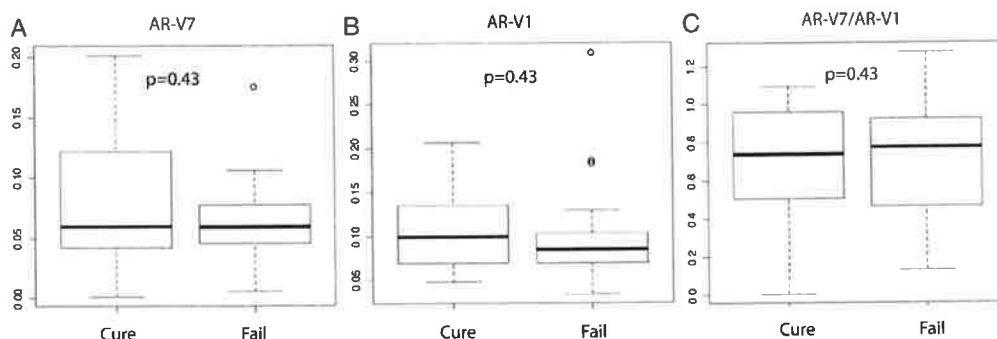


Figure 3. AR-V7 (A), AR-V1 (B) and AR-V7/V1 ratio (C) in patients cured after radical prostatectomy and those with biochemical recurrence (Fail) in mixed grade cohort.

DISCUSSION

Previous studies showed that increased AR-V7 is associated with an increased risk of biochemical recurrence after radical prostatectomy in hormone naive primary PCa and with shorter survival in men with bone metastasis.²⁻⁴ For our analysis, we chose a specific cohort of men to further investigate the prognostic value of AR-V7 as well as that of AR-V1, which inhibits AR-V7 activity. In the Stanford University radical prostatectomy specimens characterized in the study by Stamey et al, the percent of grade 4/5 cancer was the most significant predictor of the risk of biochemical recurrence.⁹ The risk was directly proportional to the percent of grade 4/5 and every 10% increase in grade 4/5 conferred an approximately 10% increase in the risk of recurrence. Hence, we chose cases with 30% to 70% grade 4/5 and an indeterminate risk of recurrence of about 50% because to our knowledge no known biomarker in such cases provides additional prognostic value.

AR-V7 expression may provide prognostic value in a more heterogeneous cohort, ie Gleason score 6-10 in the study by Guo et al,³ or in a more advanced cohort, ie bone metastasis in the study by Hornberg et al.⁴ However, our results suggest that the expression of neither AR-V7 nor V1 provided prognostic information on the men most in need of better prognostic biomarkers, ie those with an intermediate percent of grade 4/5 cancer, approximating a large subset of men with Gleason score 7 cancer. Besides patient cohorts, differences in sample preparations (fresh frozen vs FFPE tissue and whole vs macrodissected tissue) and assays (quantitative reverse transcriptase-polymerase chain reaction/immunohistochemistry vs bDNA) may also have contributed to the different conclusions.

Whether TMPRSS2-ERG fusion status is prognostic is controversial.²⁰⁻²² It was not associated with recurrence in our cohort, although it was positively associated with the percent of grade 4/5 cancer, suggesting that the fusion may increase the

growth of high grade cancer. In addition, TMPRSS2-ERG status was not associated with AR-V7 and V1 expression. Previous studies suggest that AR expression and activity contribute to TMPRSS2-ERG gene fusion.^{21,23} Although these studies did not distinguish full-length vs ligand independent AR variants, our results suggest that it is unlikely that AR variants have a major role in this process in primary PCa.

Perhaps our most interesting finding is that AR-V1 was expressed at a higher level in grade 3 only cancer compared to grade 3 cancer with associated grade 4/5 cancer. This supposedly leads to lower AR-V7 function, ie lower androgen independent transcriptional activity, in grade 3 only cancer, suggesting decreased capacity for androgen independent growth compared to that of grade 3 cancer with associated grade 4/5 cancer.

In general, little information exists on whether grade 3 cancer in mixed grade tumors differs from grade 3 only tumors. Some evidence suggests that grade 3 cancer in mixed grade tumors gives rise to grade 4/5 and, therefore, it differs from grade 3 only cancer, which never progresses to high grade cancer.²⁴ Our results support the notion that they may be different, at least in gene expression. This presents the possibility of predicting the presence of grade 4/5 cancer using biomarkers in associated grade 3 cancer in biopsies when grade 4/5 cancer is missed by sampling error. This could be helpful in clinical settings to determine a treatment strategy since men with grade 3 only tumors are essentially cured by radical prostatectomy⁹ or they may not need treatment at all, whereas high grade cancer may warrant more aggressive therapy.^{9,25}

AR-V7 expression was inversely associated with preoperative serum PSA in the grade 3 only and mixed grade cohorts, consistent with the previous finding that human PCa metastases that express higher AR-V7 expression show significantly lower PSA expression.⁴ A possible explanation is that ex-

pression is differentially regulated by androgen. AR-V7 expression is increased by androgen deprivation and decreased upon androgen restoration.⁵ On the other hand, PSA is induced by androgen and repressed by androgen deprivation.²⁶ This may also explain the observation that AR-V7 expression was positively associated with age in grade 4/5 cancer. A progressive decrease in androgen production is common after middle age,²⁷ which may lead to increased AR-V7 expression. Alternatively, higher AR-V7 levels in older patients may be due to the deregulation of alternative splicing associated with aging.²⁸

Overall, our study reveals that levels of AR-V7, AR-V1 or AR-V7/V1, or the presence of the TMPRSS2-ERG gene fusion are not predictive of recurrence in patients at indeterminate risk, underscoring the challenge of identifying prognostic factors for these patients. The diversity and complexity of human AR splice variants are well appreciated but not fully

understood. For example, multiple AR variants exist and are constitutively or conditionally active.^{29,30} Future studies must focus on better understanding the aggregate effects of full-length AR and its variants, which may together be relevant to PCa diagnosis, prognosis or treatment.

CONCLUSIONS

Regardless of TMPRSS2-ERG fusion status, neither the best characterized variant AR-V7 nor its negative regulator AR-V1 was associated with biochemical recurrence in a cohort of men at indeterminate risk for progression. The significantly higher AR-V1 expression in grade 3 only cancer compared to that in grade 3 cancer with concurrent grade 4/5 cancer points to the possibility of predicting associated grade 4/5 cancer using biomarkers in biopsies that reveal only grade 3 cancer due to sampling error.

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EDITORIAL COMMENT

Using bDNA assay, these authors examined the mRNA levels of the AR splice variants AR-V1 and V7 in a carefully selected group of paraffin embedded radical prostatectomy specimens. AR splice variants are mainly implicated in CRPC but they can be detected in untreated cases. Although this study did not examine CRPC, it provides a few informative new findings in the hormone naïve setting. Notably, AR-V levels were positively associated with age but negatively associated with preoperative serum PSA, possibly reflecting decreased PSA in elderly men and supporting the negative regula-

tion of AR-V expression by androgens.¹ The main conclusion of the study, that AR-V transcript levels are not associated with Gleason grade or PSA recurrence, seems to differ from the findings of others (references 2 to 4 in article). More studies are needed and continued efforts to examine expression levels in situ will provide more insight.

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REPLY BY AUTHORS

Our study differed in several important ways from previous studies, making direct comparisons difficult. 1) Our cases were selected from the Stamey et al series of radical prostatectomy specimens, which are more thoroughly characterized than those in most other studies.¹ These cases permit rigorous comparison of the prognostic value of candidate biomarkers, such as AR splice variants, to precisely quantitated histomorphological variables. In such evaluations biomarkers with prognostic value independent of Gleason score, a rela-

tively weak prognostic indicator, often fail to retain prognostic value compared to the percent of Gleason grade 4 + 5, a strong prognostic indicator. 2) We focused on men with high grade cancer, who have a relatively poor prognosis but still need additional risk indicators. Although AR-V1 and V7 expression did not provide this additional information, it is likely that AR splice variants have an important biological role and must be considered part of the complex androgen signaling axis in PCa.

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RESEARCH

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Patient-derived tissue slice grafts accurately depict response of high-risk primary prostate cancer to androgen deprivation therapy

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Abstract

Background: Effective eradication of high-risk primary prostate cancer (HRPCa) could significantly decrease mortality from prostate cancer. However, the discovery of curative therapies for HRPCa is hampered by the lack of authentic preclinical models.

Methods: We improved upon tumorgraft models that have been shown to predict drug response in other cancer types by implanting thin, precision-cut slices of HRPCa under the renal capsule of immunodeficient mice. Tissue slice grafts (TSGs) from 6 cases of HRPCa were established in mice. Following androgen deprivation by castration, TSGs were recovered and the presence and phenotype of cancer cells were evaluated.

Results: High-grade cancer in TSGs generated from HRPCa displayed characteristic Gleason patterns and biomarker expression. Response to androgen deprivation therapy (ADT) was as in humans, with some cases exhibiting complete pathologic regression and others showing resistance to castration. As in humans, ADT decreased cell proliferation and prostate-specific antigen expression in TSGs. Adverse pathological features of parent HRPCa were associated with lack of regression of cancer in corresponding TSGs after ADT. Castration-resistant cancer cells remaining in TSGs showed upregulated expression of androgen receptor target genes, as occurs in castration-resistant prostate cancer (CRPC) in humans. Finally, a rare subset of castration-resistant cancer cells in TSGs underwent epithelial-mesenchymal transition, a process also observed in CRPC in humans.

Conclusions: Our study demonstrates the feasibility of generating TSGs from multiple patients and of generating a relatively large number of TSGs from the same HRPCa specimen with similar cell composition and histology among control and experimental samples in an *in vivo* setting. The authentic response of TSGs to ADT, which has been extensively characterized in humans, suggests that TSGs can serve as a surrogate model for clinical trials to achieve rapid and less expensive screening of therapeutics for HRPCa and primary CRPC.

Keywords: Prostate cancer, Androgen deprivation therapy, Tumorgrafts

Background

Mortality from prostate cancer (PCa) is confined to those men who have either advanced disease (distant metastases at initial presentation) or high-risk localized PCa (HRPCa) [1,2]. The definition of HRPCa is either a Gleason score of 8–10, pre-treatment serum prostate-specific antigen (PSA) > 20 ng/ml, or clinical stage of T3/T4 at diagnosis [3]. In addition, patients with at least two of the following criteria - a Gleason score of 7, pre-

treatment serum PSA > 10 ng/ml, and a clinical stage of T2b/c - may also be considered high-risk [3]. Since < 5% of patients with newly diagnosed PCa have advanced metastatic disease, HRPCa, which comprises 15–40% of the overall PCa patient population, has become an important focus of novel therapeutic development [4,5].

Androgen receptor (AR) signaling plays a central role in all stages of PCa. For HRPCa, the standard of care is either radical prostatectomy or radiation therapy combined with androgen-deprivation therapy (ADT) [5–8]. In spite of treatment, up to 50% of these high-risk patients will inevitably progress to castration-resistant prostate

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cancer (CRPC), which is incurable, within 10 years [6,9-11]. One of the key mechanisms of resistance to ADT is the continued expression of AR by most CRPC and dependence on AR for growth [7-9,12]. Moreover, ADT induces epithelial-mesenchymal transition (EMT), a process that has been associated with aggressive clinical behavior in human PCa [13,14].

New primary therapies that can be used to eradicate HRPc alone or in combination with ADT before CRPC arises, or to treat primary CRPC effectively after ADT, are urgently needed. Because experimental models of primary human PCa are extremely limited, new generations of compounds targeting AR signaling at different levels as well as other essential pathways in PCa have been developed using pre-clinical models of metastatic CRPC [15-18]. Whether these agents will be effective against primary HRPc and derivative CRPC is not known. A realistic and representative *in vivo* model of HRPc and primary CRPC is critical for pre-clinical assessment and comparison of different treatment options. Such a model will not only accelerate the discovery of effective therapies by minimizing the number of costly and time-consuming clinical trials, but also help enhance our understanding of mechanisms of therapeutic resistance.

Remarkable correlations between drug activity in "tumorgrafts" derived directly from patient tissues and clinical outcomes have been observed [19,20]. For instance, Hidalgo et al. demonstrated a notable correlation between drug activity in patient-derived tumorgrafts and clinical outcome in 14 types of advanced cancers [20]. Multiple groups have reported the ability to establish PCa tumorgrafts in mice under the skin or renal capsule, often through the use of minced pieces of tissue [21-25]. When minced fragments of tissue are used to generate grafts, it is impossible to know the composition of any given fragment (or even whether it contains cancer), due to the heterogeneous nature of prostate tissue. This, in turn, makes it impossible to ensure that tissues with similar composition are used in control and experimental groups, which, in turn, confounds interpretation of results. In addition, it is difficult to generate enough grafts from a single prostatectomy specimen to carry out experiments to test drugs with sufficient statistical power. Unfortunately for PCa research, metastatic tissue is also very difficult to obtain, and access to such tissue is predominantly limited to the few academic programs that support "rapid autopsy" programs. For all of the reasons stated above and more, tumorgrafts of PCa are not often included in studies such as that described above by Hidalgo et al. with multiple types of cancers (but no PCa) [20].

We developed methodology to establish tumorgrafts from thin, precision-cut tissue slices of human PCa to overcome at least some of the problems [26]. This novel

in vivo tissue slice graft (TSG) model: 1) retains PCa histopathology, allowing for analysis of almost all of the cell types present in PCa and their interactions; 2) provides accurate assessment of the effects of interventions when tissues from the same specimen with similar cell composition are used as control and experimental samples; 3) ensures sufficient samples obtained for large experiments; and 4) permits optimal exchange of nutrients, oxygen, and drugs between TSG and the host.

Here we characterized TSGs generated from 6 HRPc cases as well as the castration-resistant cancer that remained in TSGs from 3 of 5 cases after ADT. We focused on high-grade components of the tumors as the likely cause of recurrence and/or castration-resistance after primary therapy. The main questions we addressed were whether cancers in TSGs maintained in intact mice retained the histology and biomarker expression of parent tumors, and whether androgen deprivation affected cell proliferation, AR-regulated gene expression and EMT of cancers in TSGs similarly to that in humans. We provide evidence that TSGs are the first realistic model of primary HRPc and CRPC that can be used with high predictive power to evaluate an exponentially growing number of molecularly targeted therapies and to discriminate the most effective therapeutics for further clinical development.

Methods

Ethics statement

All animal studies were approved by the Stanford Administrative Panel on Laboratory Animal Care (APLAC) and done in compliance with the regulations for animal studies at Stanford University. Patient-derived tissues were obtained immediately after surgery under a protocol approved by the Stanford Institutional Review Board. The participants provided their written informed consent to participate in this study.

Patient samples

Clinical and histopathologic parameters of the donors are summarized in Table 1. None of the patients had chemical, hormonal, or radiation therapy prior to radical prostatectomy.

Precision-cutting and subrenal implantation of tissue slices

Male recombination activating gene-2 (RAG2)^{-/-}γC^{-/-} mice bred at Stanford University or NIH III mice (Charles River, Wilmington, MA, USA) between 6 and 8 weeks of age were used. All animal studies were done in compliance with regulations at Stanford University. Precision-cutting and subrenal implantation of tissue slices were described previously [26]. A 25-mg testosterone pellet with a release rate of 0.2 mg/day was

Table 1 Clinical and histopathologic variables of tissue donors

ID	Pre-op PSA ng/ml	Gleason score		Clinical stage	Pathological stage	SV ¹	SM ²	ECE ³
		Biopsy	Prostatectomy					
HRPCa-1	38.48	4+3	4+3	cT1c	pT3bN0	+	-	+
HRPCa-2	8.5	4+4	4+5	cT1c	pT3bN1	+	+	+
HRPCa-3	4.6	4+3	3+4	cT2b	pT3aN0	-	+	+
HRPCa-4	36	4+3	4+4	cT2b	pT3bN1	+	+	+
HRPCa-5	6.07	4+5	3+4	cT2b	pT2cN0	-	-	-
HRPCa-6	2.09	4+5	3+4	cT2b	pT2bN0	-	-	-

¹ seminal vesicle invasion.

² surgical margin.

³ extracapsular extension.

inserted into a small incision made under the skin between the shoulder blades.

Castration of mice

Castration of mice was performed one month after subrenal implantation as previously described [26].

Immunohistochemistry

Immunohistochemistry was performed as previously described [26]. Antigen retrieval was achieved by heating in citrate buffer (pH 6.0) for 20 minutes, followed by a 20-minute cool-down. The sources and dilutions of the antibodies used in this study are listed in Table 2.

Table 2 Antibodies used in the study

Name	Source	Dilution
Anti-cytokeratin 18 (K18)	Santa Cruz Biotechnology, Santa Cruz, CA	1:200
Anti-AR	BD Pharmingen, San Diego, CA	1:200
Anti-PSA	Santa Cruz Biotechnology	1:100
Anti-AMACR/p63	Biocare, Concord, CA	ready to use
Anti-Ki67	Biocare	1:100
Anti-Ku70	Abcam, Cambridge, MA	1:200
Anti-human specific-CD31	Dako Corp., Carpinteria, CA	1:20
Anti-CCNA	Leica Microsystems, Buffalo Grove, IL	1:50
Anti-ERG	Epitomics, Burlingame, CA	1:100
Anti-VIM	Epitomics	1:100
Anti-TOP2A	Dako Corp	1:50
universal biotinylated horse anti-mouse/rabbit IgG	Vector Laboratories Inc., Burlingame, CA	1:1000
Alexa 488 goat anti-mouse	Invitrogen, Carlsbad, CA	1:1000
Alexa 555 goat anti-rabbit	Invitrogen	1:1000

Quantitation of Ki67 and CCNA expression

The proliferation index, defined as percentage of proliferating cells, was established by dividing the number of Ki67-positive cells by the total area of cancer (based on histology and/or AMACR expression) in ten 20X-microscopic fields, randomly chosen for each TSG. Similarly, the percentage of CCNA-positive cells was determined by dividing the number of CCNA-positive cells by the total area of cancer in five 20X-microscopic fields for each TSG. Student's t-test with a significant level set at $\alpha < 0.05$ was performed to determine statistical significance.

Results

Generation of TSGs from HRPCa

We generated 6–10 TSGs from each of 6 fresh HRPCa tissues obtained immediately following radical prostatectomy (Tables 1 and 3). Since high-grade cancer is likely the cause of recurrence after primary therapy, we excised tissues from areas containing mainly Gleason grade 4 and/or 5 cancer based on the ultrasound-guided prostate needle biopsy map obtained prior to surgery. While cutting at 300- μ m, every other slice was frozen, sectioned, and stained with H&E to confirm histopathology. Only slices that were in-between two slices containing high-grade PCa were implanted in mice.

Table 3 Number of TSGs generated for each HRPCa case

	Control TSGs	Castrated TSGs	Control TSGs containing cancer	Castrated TSGs containing cancer
HRPCa-1	10 ¹	NA	10	NA
HRPCa-2	5	3	5	3
HRPCa-3	3	3	3	3
HRPCa-4	5	5	5	3
HRPCa-5	4	5	4	0
HRPCa-6	4	4	4	0

¹TSGs were harvested one month after implantation. All other TSGs were harvested two months after implantation.

We first compared TSGs derived from the same parent tumor, HRPcA-1, but maintained in two mouse strains, RAG2^{-/-}γc^{-/-} [27,28] and NIH III [29,30]; both lack T, B, and natural killer cells but the extent of the deficiencies in each has not been established. Although the gross

appearance of TSGs maintained in both hosts for 1 month was similar (Figure 1A), the average graft weight and the proliferation index of cancer in RAG2^{-/-}γc^{-/-} mice were significantly higher than in NIH III mice (Figure 1B). Serial sections were stained with antibodies

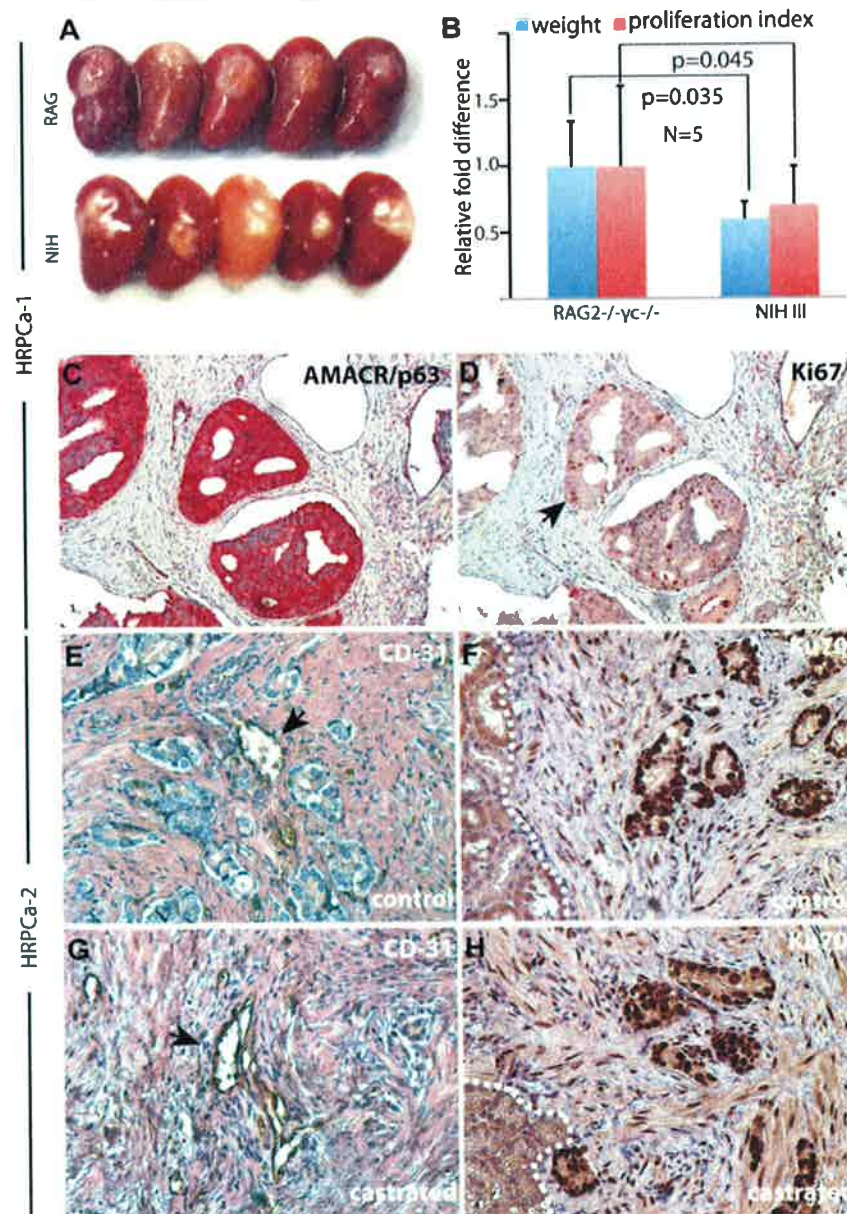


Figure 1 Generation of TSGs from HRPcA-1 and -2. (A) Kidneys with TSGs under the capsule harvested from RAG2^{-/-}γc^{-/-} or NIH III mice; (B) both the graft weight and proliferation index of TSGs maintained in RAG2^{-/-}γc^{-/-} mice were significantly higher than those maintained in NIH III mice by Student's t-test; (C-H) immunohistochemistry of TSGs in RAG2^{-/-}γc^{-/-} mice using antibodies against AMACR/p63 (C), Ki67 (D), human-specific CD31 (E and G), and human-specific Ku70 (F and H). (E-F) are control TSGs and (G-H) are TSGs one month after castration. Arrows in (D) and (E and G) point to Ki67- and CD31-positive cells, respectively. White dotted lines in (F) and (H) mark the boundary between mouse kidney and TSG. Magnification for (C) and (D) is 10X and (E-H) is 40X.

against AMACR to identify cancer cells and Ki67 to label proliferating cells (Figure 1C-D). The proliferation index in TSGs maintained in RAG2^{-/-}γc^{-/-} mice was 97% of the parent tumor. These results suggest that RAG2^{-/-}γc^{-/-} mice provide a more supportive environment for TSGs derived from HRPcCa than NIH III mice and should be the host of choice for human PCa tumorigrafts.

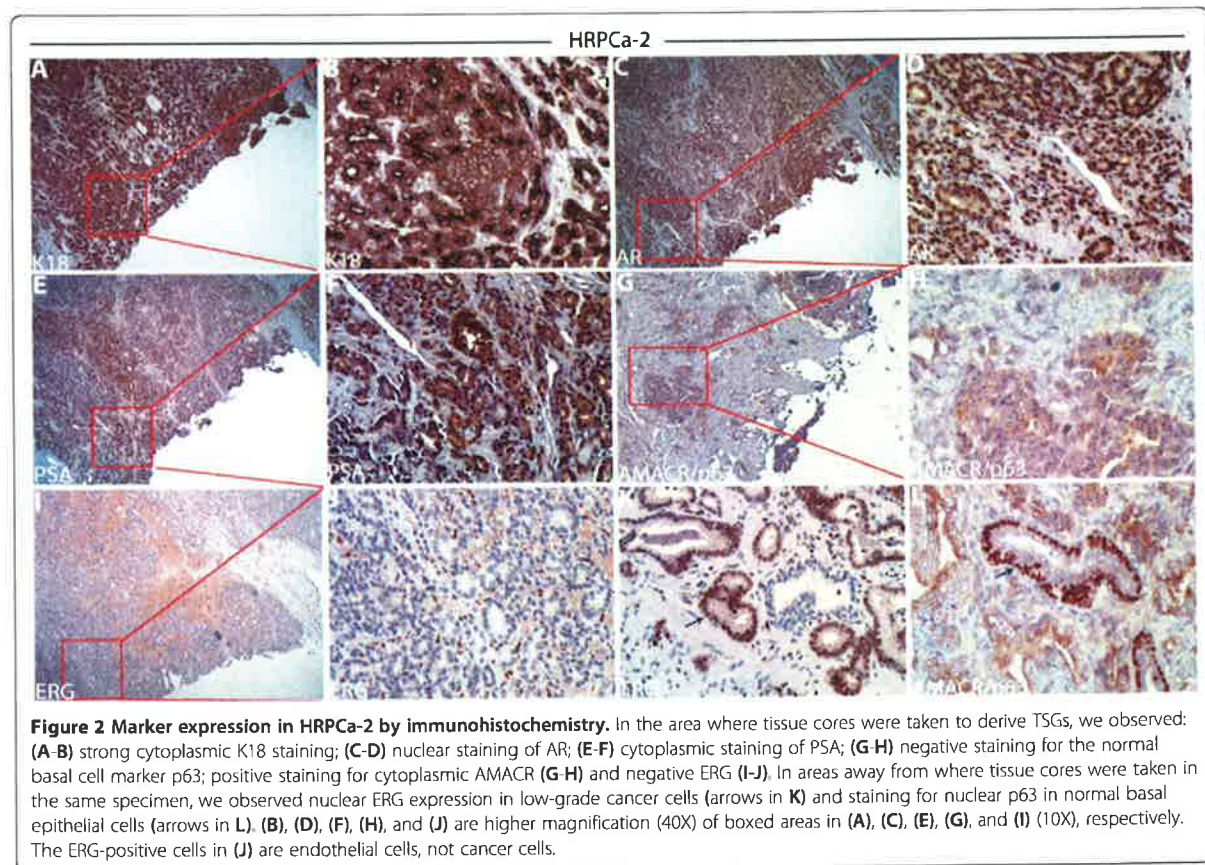
Immunohistochemistry using human-specific CD31 antibody demonstrated that a considerable amount of the vasculature present in HRPcCa TSGs two months following implantation was lined by endothelial cells of human origin, consistent with previous findings [25,26,31]. Representative images from HRPcCa-2 are shown in Figure 1E. In addition, most of the stromal cells in TSGs are of human origin as demonstrated by labeling with an antibody against human-specific nuclear antigen Ku70 (Figure 1F). Similar results were observed in TSGs derived from the same parent tumor one month after castration (Figure 1G-H), demonstrating the persistence of human endothelial and stromal cells in prostate TSGs.

TSGs derived from HRPcCa resembled the parent tumors

We compared the histology and protein expression of cell type-specific markers in TSGs to the parent tumors.

HRPcCa-2, a parent tumor, expressed classic secretory cell markers including cytoplasmic K18 (Figure 2A-B), nuclear AR (Figure 2C-D), and cytoplasmic PSA (Figure 2E-F). In contrast, the tumor was negative for the basal epithelial cell marker p63 (Figure 2G-H). Moreover, the area where the tissue was taken for TSG generation was positive for AMACR (Figure 2G-H) but negative for ERG (Figure 2I-J), two markers widely used to identify PCa in humans. AMACR is expressed by ~90% of PCa [32] and is used to identify PCa in clinical specimens. Expression of ERG is highly correlated with the presence of the TMPRSS2-ERG gene fusion present in ~50% of PCa and is negatively correlated with Gleason score (i.e., high-grade PCa is less likely to express ERG) [33]. Consistent with the negative correlation between ERG expression and Gleason score, we observed ERG expression in grade 3 cancer in the same prostatectomy specimen (Figure 2K) as well as p63 staining in normal basal cells (Figure 2L), demonstrating that absence of staining in the area where the tissue was taken was not attributable to technical failure.

TSGs derived from HRPcCa-2 harvested two months after implantation showed similar histomorphology to the parent tumor. Specifically, high-grade cancer was



readily identifiable in these TSGs, appearing as an irregular mass of neoplastic cells with little or no gland formation (Figure 3). In addition, TSGs displayed similar expression of cell-type specific markers to the parent tumor (Figure 3A-J). TSGs from the other five HRPc specimens also displayed similar histomorphology and marker expression as their parent tumors (Table 4). For example, both HRPc-1 and its derived TSGs were strongly positive for ERG (Figure 3K-L) and AMACR (Figure 3M-N). Overall, these results demonstrated that high-grade cancer from HRPc maintained appropriate histomorphology and protein expression in TSGs up to 2 months post-implantation.

Adverse pathological features of parent tumors predicted response of TSGs to ADT

Of the 6 cases used in this study, ADT was performed in 5 cases since HRPc-1 was used for comparison of host mouse strains only. Two of the cases (HRPc-5 and -6) were down-graded on final pathology of the radical prostatectomy specimens to Gleason score 7 from 9 and had no adverse pathological features such as positive surgical margin, seminal vesicle invasion, or extracapsular extension (Table 1). For accurate assessment effects of ADT, we assigned mice bearing TSGs derived from adjacent tissue slices into control and ADT groups. Interestingly, one month after ADT, cancer cells were found in 60-100% of the TSGs derived from HRPc-2, -3, and -4 (Table 3). These TSGs were defined as castration-resistant TSGs (CR-TSGs). In contrast, no cancer cells were detected in TSGs derived from HRPc-5 and -6, demonstrating complete tumor regression after ADT (Table 3). For example, TSGs derived from HRPc-3 expressed a high level of AMACR in both control and castrated mice (Figure 4A-B), whereas AMACR-expressing cancer cells were only observed in TSGs derived from HRPc-6 maintained in control but not castrated mice (Figure 4C-D). TSGs were sectioned throughout to confirm complete pathologic response to ADT (absence of AMACR-stained cells and no recognizable cancer by histopathological analysis). These results mimicked the heterogeneous response of HRPc in patients to ADT, and suggest that adverse features of parent tumors may predict the response of TSGs to ADT.

ADT modulated AR-regulated genes in TSGs similarly to PCa in humans

After ADT, remaining cancer cells in CR-TSGs derived from HRPc-2 demonstrated similar histological features and biomarkers to those in control TSGs, including expression of AR (Figure 3O-P). As expected, these cells showed little or no staining for PSA (Figure 3Q-R), consistent with the response of PCa in humans to ADT [34]. Cancer cells were also positive for K18 (Figure 3S-

T) and AMACR (Figure 3U-V), and negative for ERG (Figure 3W-X). These results suggest that ADT abolished PSA expression in cancer cells in CR-TSGs derived from HRPc but did not affect expression of other markers.

We next examined the expression of AR-regulated genes that have been reported to be up-regulated in human CRPC including TOP2A and CCNA [35]. In CR-TSGs derived from HRPc-4, the expression level and percentage of TOP2A-positive cells was significantly higher compared to control (Figure 5A-B). ADT also increased the percentage of CCNA-expressing cancer cells in CR-TSGs by 2.4-fold compared to control (Figure 5C-D, 5G). These results demonstrated that TSGs derived from HRPc responded to ADT by upregulating AR target genes associated with CRPC in humans. Moreover, the number of Ki67-expressing cells in CR-TSGs was significantly lower than that in control (Figure 5E-G), consistent with the observation that ADT inhibits cell proliferation in PCa in humans [36]. Few or no apoptotic cells were detected in CR-TSGs by cleaved caspase-3 staining (data not shown); if apoptosis was induced by ADT, it may have occurred rapidly after castration and diminished by one month after castration. Similar results were observed for HRPc-2 (Figure 5H) and -3 (Figure 5I). Together, these findings demonstrated an authentic response to ADT of HRPc in TSGs similar to that occurs in humans, suggesting that CR-TSGs realistically model primary CRPC.

ADT induced EMT in CR-TSGs similarly to human PCa

To determine the effects of ADT on EMT in TSGs and compare to its effects in PCa in humans, we examined EMT marker expression in three human prostates removed by radical prostatectomy following neoadjuvant ADT (Flutamide or Lupron treatment of 6 to 10 weeks in duration) and two untreated specimens as controls. In untreated PCa, high-grade cancers of Gleason patterns 4 and 5 showed strong cytoplasmic expression of K18 (Figure 6A), a classic epithelial cell marker. Expression of VIM, a mesenchymal marker whose expression is increased in epithelial cells during EMT, was only observed in stromal cells (Figure 6B) and mutually exclusive from K18 expression (Figure 6D). In contrast, a small population of high-grade PCa cells expressed both K18 (Figure 6E) and VIM (arrows in Figure 6F and 6H) in specimens treated with ADT. Although VIM-positive, these cells still maintained epithelial cell morphology, i.e., cuboidal rather than spindle-shaped. These results suggest that a subset of high-grade prostate cancer cells in humans treated with ADT underwent EMT.

A similar staining pattern was observed in CR-TSGs derived from HRPc-2, -3 and -4. Specifically, a rare

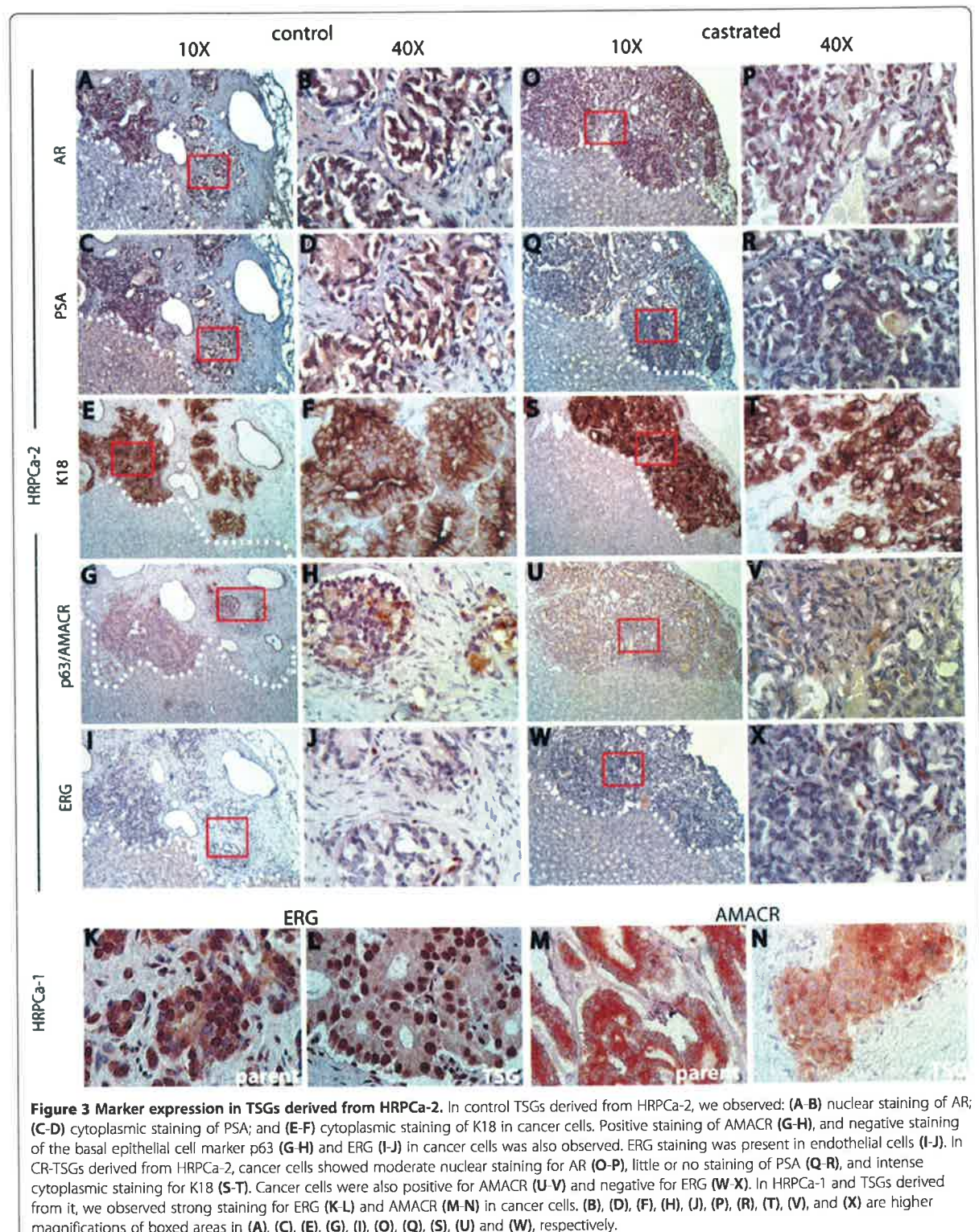


Table 4 Marker expression in HRPc where tissues were taken for TSG generation and in corresponding TSGs

ID	K18	AR	PSA	AMACR	P63	ERG
HRPCa-1	+	+	+	+	-	+
Derivative TSGs	+	+	+	+	-	+
HRPCa-2	+	+	+	+	-	-
Derivative TSGs	+	+	+	+	-	-
HRPCa-3	+	+	+	+	-	-
Derivative TSGs	+	+	+	+	-	-
HRPCa-4	+	+	+	+	-	-
Derivative TSGs	+	+	+	+	-	-
HRPCa-5	+	+	+	-	-	-
Derivative TSGs	+	+	+	-	-	-
HRPCa-6	+	+	+	+	-	-
Derivative TSGs	+	+	+	+	-	-

population of cancer cells expressing both VIM and K18 was observed in CR-TSGs (Figure 6M-P) but not in control TSGs (Figure 6I-L). Moreover, E-cadherin, a well-known epithelial marker, was primarily localized on the cell membrane in cancer cells of control TSGs, while in CR-TSGs, it was mislocalized away from the cell membrane into the nucleus (Figure 7). This leads to loss of function of E-cadherin, commonly observed during EMT [37,38]. These results suggest that ADT induced EMT in the TSG model similar to CRPC in humans.

Discussion

We have shown that TSGs derived from HRPc recapitulate characteristics of parent tumors including histopathological features, biomarker expression, and responses to ADT. Our study differs from previous reports [21-26] of generation of prostate tumorgrafts in several ways. First, we implanted precision-cut tissue slices rather than minced tissues as used in previous studies. The ability to reliably determine the presence and grade of cancer prior to implantation is one of the advantages of this methodology, which in turn allows more accurate assessment of therapeutic effects in large-scale animal trials. For example, our study design ruled out the possibility that "bad" tumors were missed in the two cases that showed complete regression after castration. Because we used only slices that were in-between two slices containing high-grade PCa and assigned adjacent tissue slices to control and ADT groups, it is unlikely that the "bad" tumors were missed only in the ADT but not the control group. If minced tissues were used without prior knowledge of histopathology, it would be impossible to know whether the complete regression was just by chance, i.e., the bad tumors were missed or there was no cancer present initially in the ADT group. Second, we focused on high-risk tumors, the major contributor to PCa mortality, rather than benign or low-risk tumors that have been used in most previous studies. Third, we systematically evaluated the effects of ADT, a standard treatment for HRPc, in our

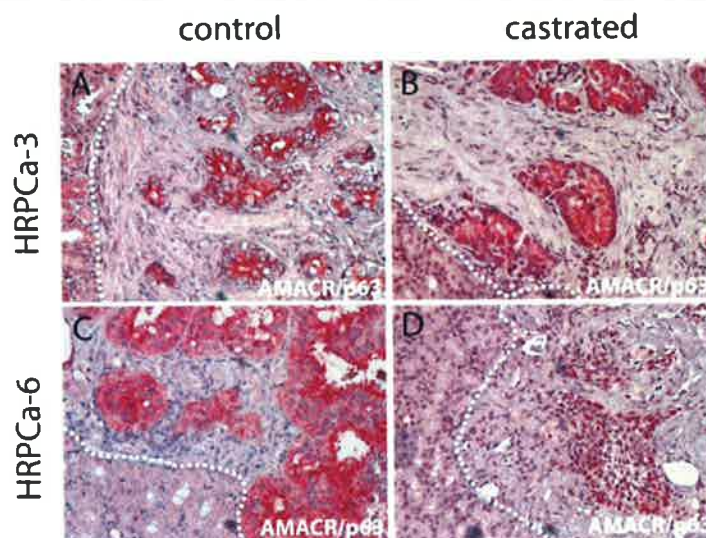


Figure 4 Differential responses to ADT of TSGs derived from HRPc with or without adverse pathological features.

Immunohistochemistry using an antibody cocktail for cytoplasmic AMACR and nuclear p63 demonstrated resistance of HRPc-3, a HRPc with adverse pathological features, to ADT (A-B), and complete tumor regression in HRPc-6, a HRPc without adverse pathological features, after ADT (C-D). In (D), remnant epithelial cells are benign, as demonstrated by nuclear p63 staining. White dotted lines mark the boundary between mouse kidney and TSG. Magnification for all images is 40X.

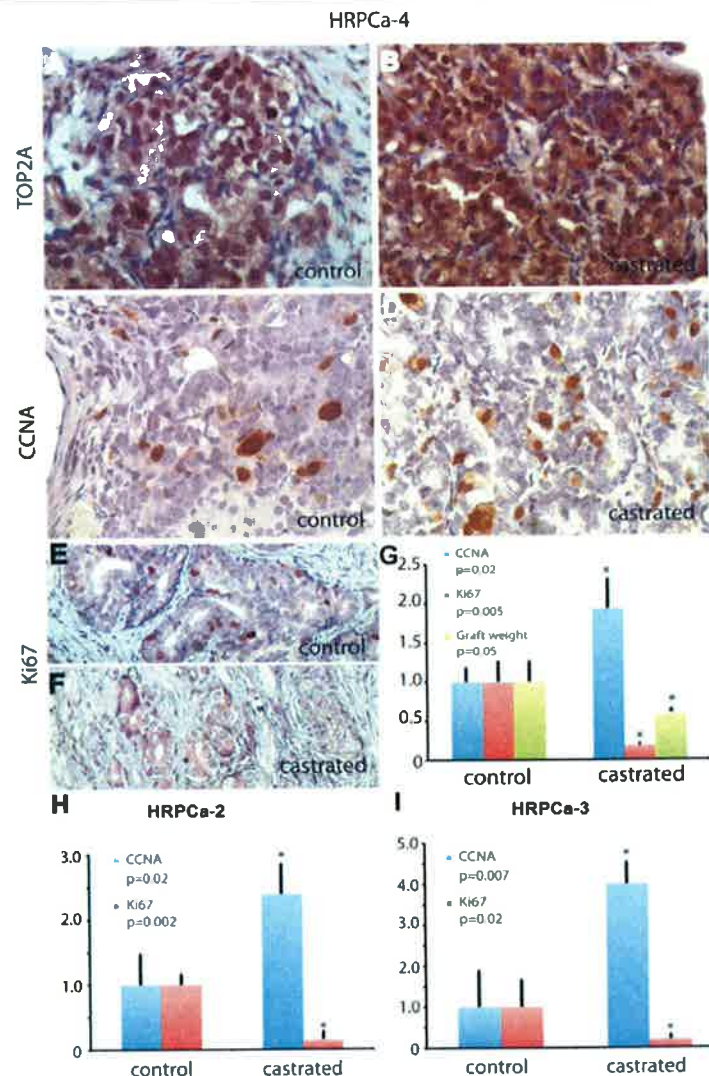


Figure 5 Effects of ADT on AR target genes, cell proliferation, and graft weight in HRPc-4 TSGs. ADT dramatically increased the expression of TOP2A in CR-TSGs (B) compared to control (A). Similarly, the number of CCNA-positive cells was 2.4-fold higher in CR-TSGs (D) than in control TSGs (C). Ki67-positive cells were dramatically decreased in CR-TSGs (F) compared to control TSGs (E). This decrease was statistically significant as was the decrease in graft weight in response to ADT by Student's t-test (G). ADT also decreased cell proliferation and upregulated CCNA expression in HRPc-2 (H) and HRPc-3 (I). The values in castrated TSGs were normalized against control. * marks significant difference between castrated and control TSGs defined as $p < 0.05$ by Student's t-test.

TSGs. Although the number of cases in our study is small, it is the largest cohort of HRPc evaluated for response to ADT to date in a preclinical model.

HRPc is the target of adjuvant and neoadjuvant therapies since low-risk PCa is largely curable by surgery or radiation or needs no treatment. A growing inventory of new agents has been discovered that may improve the clinical outcome of HRPc. Clinical trials evaluating such candidate compounds require a large number of patients,

are expensive and time-consuming, and expose patients to certain risks. The TSG model of HRPc provides a much-needed pre-clinical screening platform that can be used to rapidly narrow down the number of agents or regimens for further investigation in clinical trials. The authenticity of the model in recapitulating the features of the parent tumors increases confidence in the likelihood of similar drug responses in humans. In addition, our study demonstrates the feasibility of generating a relatively large

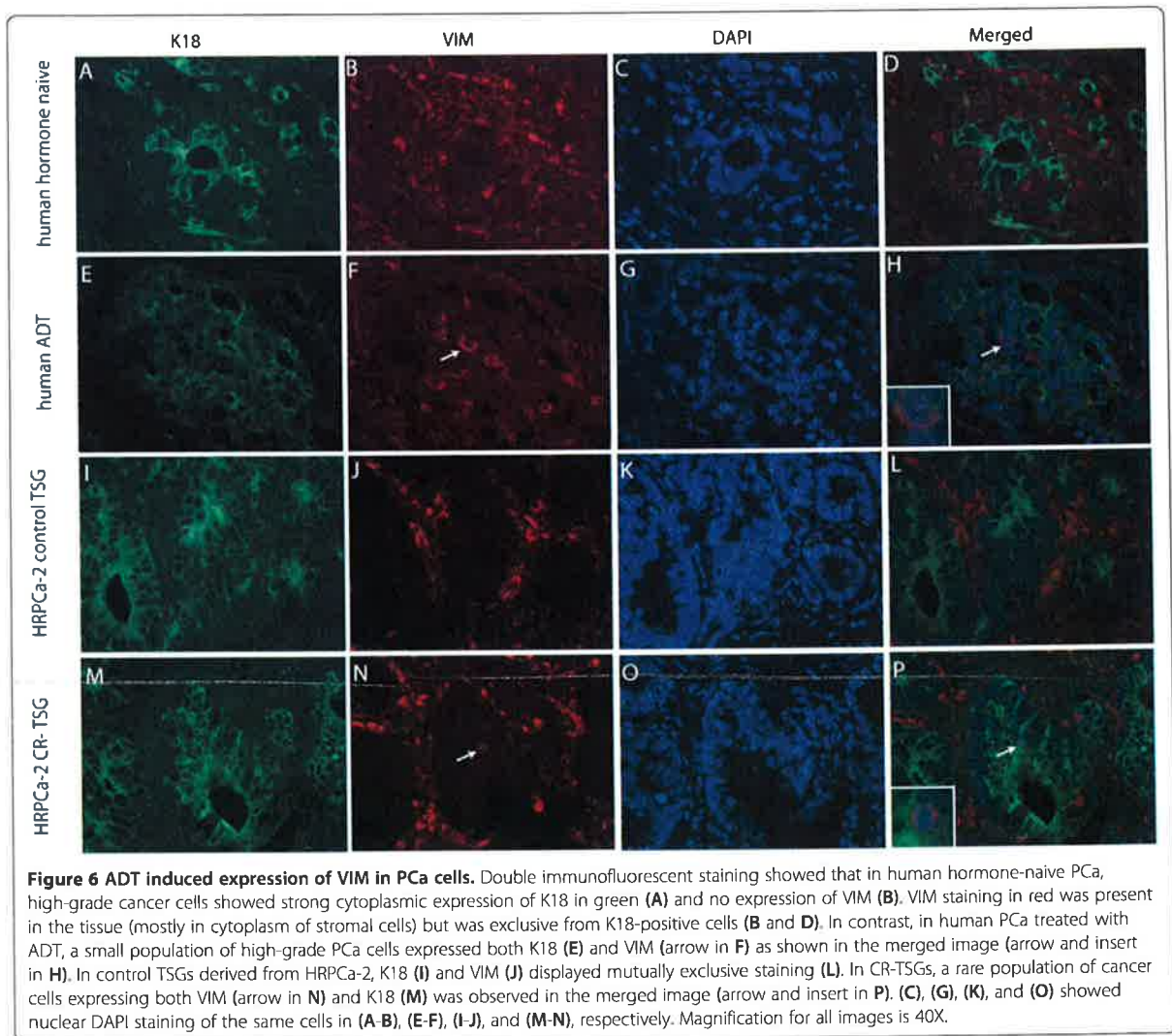


Figure 6 ADT induced expression of VIM in PCa cells. Double immunofluorescent staining showed that in human hormone-naïve PCa, high-grade cancer cells showed strong cytoplasmic expression of K18 in green (A) and no expression of VIM (B). VIM staining in red was present in the tissue (mostly in cytoplasm of stromal cells) but was exclusive from K18-positive cells (B and D). In contrast, in human PCa treated with ADT, a small population of high-grade PCa cells expressed both K18 (E) and VIM (arrow in F) as shown in the merged image (arrow and insert in H). In control TSGs derived from HRPc-a-2, K18 (I) and VIM (J) displayed mutually exclusive staining (L). In CR-TSGs, a rare population of cancer cells expressing both VIM (arrow in N) and K18 (M) was observed in the merged image (arrow and insert in P). (C), (G), (K), and (O) showed nuclear DAPI staining of the same cells in (A-B), (E-F), (I-J), and (M-N), respectively. Magnification for all images is 40X.

number of TSGs from the same HRPc-a specimen with similar cell composition and histology among control and experimental samples in an *in vivo* setting. This capability is particularly useful since PCa specimens are becoming smaller due to early cancer detection. Our model can be used to test a variety of therapeutic strategies, including potential curative therapies for HRPc-a that can either prevent CRPC from arising during ADT or kill CRPC cells after disease progression. Since ADT may be associated with numerous side effects such as increased cardiovascular mortality, other alternative therapies should also be investigated [39]. Finally, our model can be used to better understand the mechanisms of development of CRPC, which will in turn accelerate the discovery of effective therapies.

As proof-of-principle, we have demonstrated that our model closely mimics the response of PCa in humans to

ADT. First, ADT decreased cell proliferation and reduced graft weight of TSGs. Second, ADT downregulated the conventional AR target gene PSA while selectively upregulating CCNA and TOP2A in CR-TSGs, as in human PCa [35], suggesting that the TSG model is a suitable platform for pre-clinical testing of the ever-growing number of new therapeutic agents that aim to better prevent AR activation in CRPC. Third, consistent with recent studies highlighting a role for EMT after ADT in facilitating human PCa progression and metastasis [14,40,41], cancer cells in CR-TSGs exhibited EMT by simultaneously expressing both mesenchymal and epithelial cell markers, VIM and K18, respectively. In addition, E-cadherin was mislocalized away from cell membranes into the nuclei in CR-TSGs, presumably disrupting the function of E-cadherin in preventing beta-catenin from entering the nucleus [38]. Such

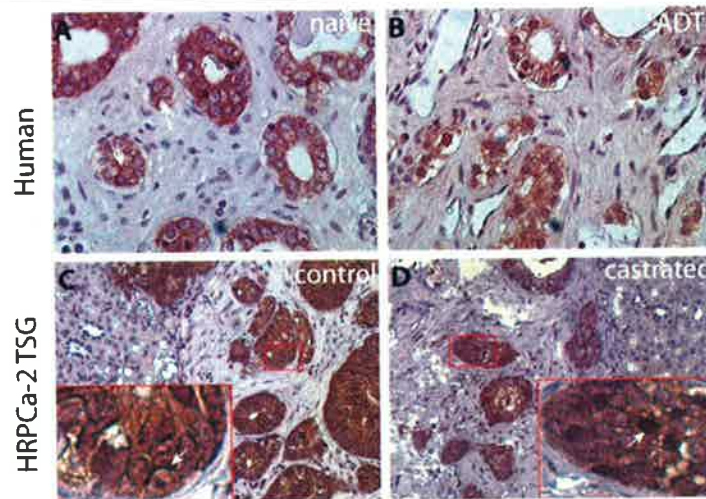


Figure 7 ADT induced mislocalization of E-cadherin in TSGs derived from HRPc-2. E-cadherin, a well known epithelial marker, was primarily localized on cell membranes in cancer cells of human hormone-naïve PCa (A) and control TSGs derived from HRPc-2 (C), while in human PCa treated with ADT (B) and castrated TSGs (D), it was mislocalized away from the cell membrane (arrow in C) into the nucleus (arrow in D). The inserts are enlarged images of the boxed area in each image. Magnification for (A) and (B) is 40X, and for (C) and (D), 20X.

mislocalization was recently observed in a metastatic colorectal cancer model in which E-cadherin nuclear translocation was associated with aggressive focal growth [42], suggesting that mislocalization of E-cadherin may be a general mechanism of cancer progression. The documentation of ADT-induced EMT in CR-TSGs derived from HRPc-2 suggests an attractive model for testing novel therapeutics aimed at blocking EMT.

Our findings are the first to link seminal vesicle invasion, positive surgical margin and extracapsular extension to lack of complete pathologic response to ADT by HRPc-2. The efficacy of neoadjuvant ADT in the TSG model appears much better than in patients determined by histology [43,44]. Since the presence or absence of tumor cells in TSGs was evaluated one month after castration, we can't rule out the possibility that the regressed tumors might relapse at later time points. In addition, most studies show a lower serum testosterone level in castrated mice than in humans [26,45-47], possibly because unlike in humans, adrenal glands in mice do not produce androgen [48-50]. Thus, castration of mice may more effectively eliminate HRPc-2 cells in TSGs than does ADT in humans. Further experiments are needed to determine the long-term effects of ADT and to investigate the possibility of serial passage in this model. Mechanisms of resistance to therapy can be explored, such as the role of stem cells in castration-resistance.

It is interesting to note that endothelial and stromal cells in TSGs are mostly of human origin, rather than replaced by their host counterparts. This is consistent with

a recent report demonstrating a burst of angiogenesis by endogenous human blood vessels in primary xenografts of benign prostate or PCa tissues that occurred between days 6–14 after transplantation into SCID mice pre-implanted with testosterone pellets [25]. In contrast, DeRose et al. demonstrated that, in human breast tumorgrafts, cancer-associated stroma and endothelial cells from the original tumor were largely replaced by mouse-derived stroma and endothelial cells [51]. This difference may be due to the disparate growth properties of these two types of cancers – PCa is a slow-growing cancer with a long natural history, whereas breast cancer is much more aggressive. The slow-growing nature of prostate TSGs perhaps makes it unnecessary to incorporate host stromal and endothelial cells in the grafts. In our study, human stromal and endothelial cells survived up to 2 months in TSGs derived from HRPc-2. It would be interesting to determine whether the human endothelial and stromal cells would eventually be replaced by their mouse counterparts in long-term follow-up of these grafts.

Conclusions

We provide evidence that TSGs are a realistic model of primary HRPc-2 and CRPC that may be used with high predictive power to evaluate the exponentially growing number of molecularly targeted therapies and to discriminate the most effective therapeutics for further clinical development.

Abbreviations

ADT: Androgen deprivation therapy; AR: Androgen receptor; CRPC: Castration-resistant prostate cancer; CR-TSGs: Castration-resistant tissue

slice grafts; EMT: Epithelial-mesenchymal transition; PCa: Prostate cancer; PSA: Prostate-specific antigen; TSG: Tissue slice graft.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HZ conceived of the study, participated in its design, carried out the TSG generation and castration, analyzed data and drafted the manuscript, AT and AI assisted with TSG generation and castration, RN, SWR, and JS carried out the immunostaining, DMP conceived of the study, and participated in its design and revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Department of Defense (grant number W81XWH-10-0576). The funding body does not have any role in the design, collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

Received: 30 May 2013 Accepted: 16 August 2013

Published: 28 August 2013

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doi:10.1186/1479-5876-11-199

Cite this article as: Zhao et al.: Patient-derived tissue slice grafts accurately depict response of high-risk primary prostate cancer to androgen deprivation therapy. *Journal of Translational Medicine* 2013 **11**:199.

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